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Insulin-like Growth Factor Binding Protein Fragments and the Utilization Thereof

The present invention relates to peptides having cell-proliferative and cell-protective properties, complexes of the peptides with human insulin-like growth factors (IGF) I and II, and the related nucleic acids, antisense nucleotides, antibodies and inhibitors.

Insulin-like growth factor binding proteins have been described, inter alia, by Shimasaki, S., and Ling, N., in Prog. Growth Factor Res. 3 (1991) 243-266, and Zapf, J., in Eur. J. Endocrinol. 132 (1995) 645-654.

The present invention relates to peptides the amino acid sequence of which corresponds to parts of the amino acid sequence of insulin-like growth factor binding proteins, and cyclic, glycosylated, phosphorylated, acetylated, amidated and/or sulfated derivatives thereof. These peptides according to the invention are designated as IGFBP or IBP.

Preferred peptides are those which are naturally occurring and can be isolated, for example, from hemofiltrate. Preferably, the peptides have lengths of from 61 to 115 amino acids. Particularly preferred are peptides having sequences which correspond to N- or C-terminal sequences of insulin-like growth factor binding proteins.

Preferred peptides are peptides having an amino acid sequence of formula

$$R_1\text{-}C\text{-}X_1\text{-}PNC\text{-}X_2\text{-}QC\text{-}X_3\text{-}CWCV\text{-}X_4\text{-}C\text{-}R_2$$

wherein

R_1 is NH_2, an amino acid or a peptide having an amino acid sequence comprising up to 41 amino acids, X_1 is a peptide having an amino acid sequence comprising from 24 to 31 amino acids, X_2 is a peptide having an amino acid sequence comprising 9 amino acids, X_3 is a peptide having an amino acid sequence comprising 10 amino acids, X_4 is a peptide having an amino acid sequence comprising from 18 to 24 amino acids, R_2 is COOH, $CONH_2$ or a peptide having up to 12 amino acids, and cyclic, glycosylated, phosphorylated, acetylated, amidated, sulfated derivatives and or fragments thereof having the physiological activity of IGFBP.

The peptide has cell-proliferative and cell-protective properties.

The peptides according to the invention can have disulfide bridges to correspond to the general formula:

$$R_1\text{-}\overline{C\text{-}X_1\text{-}PNC}\text{-}X_2\text{-}Q\overline{C\text{-}X_3\text{-}CWCV}\text{-}X_4\text{-}\overline{C}\text{-}R_2$$

In a preferred embodiment, the peptides have a glycine on one or more of the following positions of the amino acid sequence. X_2 on position 4, X_3 on position 9, X_4 on position 4 or 5, and/or X_4 on position 9 or 10.

It is further preferred that X_1 is L or V on position 8, and/or X_1 is L or I on position 11, and/or X_2 is D or N on position 1, and/or X_2 is K or R on position 9, and/or X_3 is S or A on position 3 and/or R or A on position 8.

In a particularly preferred embodiment, R_1 is selected from:

APSEEDHSILWDAISTYDGSKALHVTNIKKWKEP (SEQ ID NO: 1),
GGKHHLGLEEPKKLRPPPARTP (SEQ ID NO: 2),
GKGGKHHLGLEEPKKLRPPPARTP (SEQ ID NO: 3),
GHAKDSQRYKVDYESQSTDTQNFSSESKRETEYGP (SEQ ID NO: 4),
KVNGAPREDARPVPQGS (SEQ ID NO: 5),
LTQSKFVGGAENTAHPRIISAPEMRQESEQGP (SEQ ID NO: 6),

PQAGTARPQDVNRRDQQRNPGTSTTPSQPNSAGVQDTEMGP (SEQ ID NO: 7); and/or

X_1 is selected from

RIELYRVVESLAKAQETSGEEISKFYL (SEQ ID NO: 8),
QQELDQVLERISTMRLPDERGPLEHLYSLHI (SEQ ID NO: 9),
RREMEDTLNHLKFLNVLSPRGVHI (SEQ ID NO: 10),
QSELHRALERLAASQSRTHEDLYIIPI (SEQ ID NO: 11),
RRHMEASLQELKASPRMVPRAVYL (SEQ ID NO: 12),
RRHLDSVLQQLQTEVYRGAQTLYV (SEQ ID NO: 13); and/or

X_2 is selected from

NKNGFYHSR (SEQ ID NO: 14),
DKHGLYNLK (SEQ ID NO: 15),
DKKGFYKKK (SEQ ID NO: 16),
DRNGNFHPK (SEQ ID NO: 17),
DRKGFYKRK (SEQ ID NO: 18),
DHRGFYRKR (SEQ ID NO: 19); and/or

X_3 is selected from

ETSMDGEAGL (SEQ ID NO: 20),
KMSLNGQRGE (SEQ ID NO: 21),
RPSKGRKRGF (SEQ ID NO: 22),
HPALDGQRGK (SEQ ID NO: 23),
KPSRGRKRGI (SEQ ID NO: 24),
RSSQGQRRGP (SEQ ID NO: 25); and/or

X_4 is selected from

YPWNGKRIPGSPEIRGDPN (SEQ ID NO: 26),
NPNTGKLIQGAPTIRGDPE (SEQ ID NO: 27),
DKYGQPLPGYTTKGKEDVH (SEQ ID NO: 28),

DRKTGVKLPGGLEPKGELD (SEQ ID NO: 29),
DKYGMKLPGMEYVDGDFQ (SEQ ID NO: 30),
DRMGKSLPGSPDGNGSSS (SEQ ID NO: 31); and/or

R_2 is selected from

QIYFNVQN (SEQ ID NO: 32),
HLFYNEQQEARGVHTQRMQ (SEQ ID NO: 33),
HLFYNEQQE (SEQ ID NO: 34),
YSMQSK (SEQ ID NO: 35),
HQLADSFRE (SEQ ID NO: 36),
HTFDSSNVE (SEQ ID NO: 37),
PTGSSG (SEQ ID NO: 38).

Preferred peptides have the following sequences:

IGFBP-1
APSEEDHSILWDAISTYDGSKALHVTNIKKWKEPCRIELYRVVESLAKAQETSGEEISKFYL
PNCNKNGFYHSRQCETSMDGEAGLCWCVYPWNGKRIPGSPEIRGDPNCQIYFNVQN
(SEQ ID NO: 39)

IGFBP-2
GKGGKHHLGLEEPKKLRPPPARTPCQQELDQVLERISTMRLPDERGPLEHLYSLHIPNCDK
HGLYNLKQCKMSLNGQRGECWCVNPNTGKLIQGAPTIRGDPECHLFYNEQQEARGVHTQ
RMQ (SEQ ID NO: 40)

GGKHHLGLEEPKKLRPPPARTPCQQELDQVLERISTMRLPDERGPLEHLYSLHIPNCDKH
GLYNLKQCKMSLNGQRGECWCVNPNTGKLIQGAPTIRGDPECHLFYNEQQEARGVHTQR
MQ (SEQ ID NO: 45)

IGFBP-3
GHAKDSQRYKVDYESQSTDTQNFSSESKRETEYGPCRREMEDTLNHLKFLNVLSPRGVHI
PNCDKKGFYKKKQCRPSKGRKRGFCWCVDKYGQPLPGYTTKGKEDVHCYSMQSK (SEQ
ID NO: 41)

KVDYESQSTDTQNFSSESKRETEYGPCRREMEDTLNHLKFLNVLSPRGVHIPNCDK KGFYKKKQCRPSKGRKRGFCWCVDKYGQPLPGYTTKGKEDVHCYSMQSK (SEQ ID NO: 46)

HPLHSKIIIIKKGHAKDSQRY (SEQ ID NO: 47)

IGFBP-4

DEAIHCPPCSEEKLARCRPPVGCEELVREPGCGCCATCALGLGMPCGVYTPRCGSGLRCYP PRGVEKPLHTLMHGQGVCMELAEIEAIQESLQPSDKDEGDHPNNSFSPCSAHDRRCLQKH FAKIRDRSTSGGKM (SEQ ID NO: 48)

KVNGAPREDARPVPQGSCQSELHRALERLAASQSRTHEDLYIIPIPNCDRNGNFHPKQCHP ALDGQRGKCWCVDRKTGVKLPGGLEPKGELDCHQLADSFRE (SEQ ID NO: 42)

IGFBP-5

LTQSKFVGGAENTAHPRIISAPEMRQESEQGPCRRHMEASLQELKASPRMVPRAVYLPNC DRKGFYKRKQCKPSRGRKRGICWCVDKYGMKLPGMEYVDGDFQCHTFDSSNVE (SEQ ID NO: 43)

KFVGGAENTAHPRIISAPEMRQESEQGPCRRHMEASLQELKASPRMVPRAVYLPNCDR KGFYKRKQCKPSRGRKRGICWCVDKYGMKLPGMEYVDGDFQCHTFDSSNVE (SEQ ID NO: 49)

HTRISELKAEAVKKDRRKKLTQS (SEQ ID NO: 50)

IGFBP-6

PQAGTARPQDVNRRDQQRNPGTSTTPSQPNSAGVQDTEMGPCRRHLDSVLQQLQTEVY RGAQTLYVPNCDHRGFYRKRQCRSSQGQRRGPCWCVDRMGKSLPGSPDGNGSSSCPTG SSG (SEQ ID NO: 44).

The peptides according to the invention can be obtained by purification from human hemofiltrate or urine, by solid-phase peptide synthesis, or by expression in recombinant microorganisms.

The invention further relates to complexes of the peptides according to the invention with human insulin-like growth factor I and/or human insulin-like growth factor II and its physiologically active fragments and/or derivatives, especially amidated, acetylated, sulfated, phosphorylated and/or glycosylated derivatives.

In addition, the invention relates to nucleic acids coding for the peptides according to the invention, antisense nucleotides which, under stringent conditions, bind to a nucleic acid coding for the peptide according to the invention, antibodies which bind to the peptides according to the invention, inhibitors which inhibit the biological activity of insulin-like growth factor binding proteins, inhibitors which inhibit the expression of insulin-like growth factor binding proteins.

The peptides, complexes, nucleic acids, antisense nucleotides, antibodies and inhibitors according to the invention are especially suitable for the preparation of a medicament for treating the over- or underexpression of insulin-like growth factor binding proteins, for treating muscular atrophy, osteoporosis, diabetes, amyloid lateral sclerosis, peripheral and central neuropathies, inflammatory processes, disordered inflammatory reactions, tumor diseases, inflammatory and neoplastic diseases, disturbance of growth, muscular affections, affections of the bone system, and/or for wound and bone healing.

Especially complexes of IGFBP with IGF-I or IGF-II are useful for the treatment of bone diseases.

The peptides according to the invention and the complexes of the peptides with insulin-like growth factor have a cell-proliferative activity.

The peptides according to the invention control the release of IGF-I and IGF-II from the complexes at their site of action. The coadministration of the peptides according to the invention with IGF-I or IGF-II extends the biological half-life and thus the availability of the latter. Hypoglycemia induced by the injection of free IGF-I or IGF-II is prevented by the coadministration of the peptides according to the invention.

In addition, the peptides according to the invention have a growth-promoting effect on bone cells and lead to an enhancement or modulation of the activity of growth hormones.

In a preferred embodiment, the peptides, complexes, nucleic acids, antisense nucleotides, antibodies and inhibitors according to the invention are contained in a pharmaceutically acceptable dosage form in a medicament. They are suitable for oral, intravenous, intramuscular, intracutaneous, intrathecal administration, or as an aerosol for transpulmonary administration. The amount of peptide to be administered is from 1 µg to 1 g per dosage unit per day.

The nucleic acids and/or antisense nucleotides according to the invention are also suitable for the preparation of a medicament for the treatment of somatic or non-somatic genetic diseases.

The diagnostic agent according to the invention contains the peptides, complexes, nucleic acids, antisense nucleotides, antibodies and/or inhibitors according to the invention.

Preferably, the diagnostic agent contains poly- or monoclonal antibodies against the peptide according to the invention. Such antibodies may be fluorescence-labeled or radioactively labeled to be used in the known ELISA or RIA. However, the diagnostic agent may also contain nucleic acids which, in a modified or labeled form, are employed in test known to those skilled in the art, such as PCR or fingerprinting.

The diagnostic agents according to the invention are especially useful for diagnosing functional disorders in bones, muscles, the nervous system, lymph organs, the gastrointestinal tract, the immune system, and of diabetes and inflammatory and neoplastic processes, and as a marker in cancer.

Especially the simultaneous occurrence of several fragments of BP-4 or BP-5 in the plasma, in particular, of the N- and C-terminal domains, is useful as a marker for

diseases of bone metabolism. The corresponding peptides can be detected by mass spectroscopy, preferably by an immune reaction with corresponding antibodies.

Figure 1 shows an alignment of the consensus sequences of C-terminal fragments of the insulin-like growth factor proteins.

Figure 2 shows the schematic structure of the insulin-like growth factor proteins with the cysteine-rich N- and C-terminal domains.

Figure 3 shows the schematic structure of the insulin-like growth factor proteins and the sequence of the biologically active fragments isolated from hemofiltrate.

Figure 4 shows the isolation of the osteoanabolic factor IGFBP-4-11 from human hemofiltrate (see Example 3).

Figure 5 shows the sequence and sulfur bridge analysis of the osteoanabolic factor IGFBP-4-11. The cysteines 153-183, 194-205 and 207-228 are bridged.

Figure 6 shows the biological effect of the osteoanabolic factor IGFBP-4-11. After incubation of primary rat osteoblasts, which had been kept in a serum-free medium, for (A) 24 hours, (B) 48 hours, and (C) 72 hours with IGFBP-4-11, the proliferation-promoting effect of IGFBP-4-11 can be seen. An increase of the DNA synthetic rate in a dose-dependent way is found, measured as incorporation of bromodeoxyuridine (BrdU).

Figure 7 shows the specific binding to osteoblasts and the possible receptor for the osteoanabolic factor IGFBP-4-11 (designated as IGFBP-$4^{136-237}$). A: Radioactively labeled IGFBP-4-11 exhibits specific binding to primary osteoblast cells which can be displaced by increasing amounts of non-labeled IGFBP-4-11. B: After having bound to osteoblasts, radioactively labeled IGFBP-4-11 could be chemically cross-linked with its putative receptor molecule and subsequently be detected by gel electrophoresis followed by autoradiography. The ligand-receptor complex has a molecular mass of about 120 kDa. The formation of the complex is favored by

saponin, a membrane pore generator. Complex formation is prevented by adding an excess of unlabeled IGFBP-4-11 to the incubation mixture.

The purification of the peptide according to the invention or its complex is illustrated by the following Examples:

Example 1

Purification and peptide-chemical analysis of IGFBP-2-13

The peptides according to the invention can be obtained by a purification method starting from human hemofiltrate. This patent method (Forssmann, W.-G. (1988), German Laid-Open DE 36 33 707 A1), which was developed for the recovery of proteins from hemofiltrate, was also employed, in a modified form, for the purification of the peptide complex.

Hemofiltrate batch extraction

Hemofiltrate is obtained in large amounts in the ultrafiltration of the blood of kidney disease sufferers. 800 to 1,000 l of hemofiltrate are adjusted to pH 2.7 with HCl and diluted with water to a conductivity of 5.5 mS/cm, and applied to a strong cation-exchanger at a flow rate of 3 l/min.

Conditions of chromatography:

column:	Vantage VA 250 (Amicon, Witten)
column material:	Fractogel TSK SP 650 (M), 25 cm x 20 cm
flow rate:	3 l/min
detection:	280 nm, pH, conductivity
buffer A:	hemofiltrate, pH 2.7, conductivity 5.5 mS/cm
buffer B:	0.5 M ammonium acetate
plant:	Autopilot Chromatographiesystem (PerSeptive Biosystems, Wiesbaden)

After application of a total of 1,000 l of liquid over night, the column is washed with several column volumes of 5 mM HCl. The elution of the bound peptides is performed as a batch elution with 0.5 M ammonium acetate. Complete elution of the peptides is achieved with an increasing pH value (6.8 to 7.2) and increased conductivity (56 mS/cm) in about 5 l of eluate.

First preparative separation

The ammonium acetate eluates from the batch extraction are combined in amounts of 5,000 to 10,000 l of hemofiltrate peptide. After adjusting the pH to 2.7, the peptide extract is applied to the preparative cation-exchanger with admixing completely desalted water having a conductivity of 5.5 mS/cm.

Conditions of chromatography:

column:	Vantage 250 VA
column material:	Fractogel TSK SP 650 (M), 25 cm x 20 cm
flow rate:	up to 3 l/min during application
	0.5 to 1 l during elution
detection:	280 nm, pH, conductivity
sample:	hemofiltrate, pH 2.7, conductivity 5.5 mS/cm
plant:	Autopilot Chromatographiesystem (PerSeptive Biosystems, Wiesbaden)

After application of the raw extract over a period of 240 min, the column is washed with 0.01 M HCl until the conductivity has fallen below 1 mS/cm. Elution is effected in several steps with the following buffers:

buffer	pH value	buffer substances	conductivity (mS/cm)
washing buffer	2.0	0.01 M HCl	1
elution buffer 1	3.6	0.1 M citric acid 1-hydrate	2.9
elution buffer 2	4.5	0.1 M acetic acid + 0.1 M sodium acetate	4.0
elution buffer 3	5.0	0.1 M malic acid	6.2
elution buffer 4	5.6	0.1 M succinic acid	6.1
elution buffer 5	6.6	0.1 M NaH_2PO_4	4.9
elution buffer 6	7.4	0.1 M NaH_2PO_4	6.7
elution buffer 7	9.0	0.1 M ammonium carbonate	6.7

Eluates 1-7 are referred to as pH pool I-VII. They are separately collected and finally washed with completely desalted water. Elution is performed until a new baseline is reached. For the individual pH pools I to VII, elution volumes of 10 to 25 l are reached.

Second preparative separation

The individual pH pools are separated by reversed-phase chromatography for fractionating and desalting at the same time.

Conditions of chromatography:

column:	FineLine 100 (Pharmacia, Freiburg)
column material:	Source RPC, 15 µm, 10 x 12.5 cm (FineLine 100)
flow rate:	150 ml/min (FineLine 100)
detection:	280 nm, conductivity, pH
buffer A:	10 mM HCl
buffer B:	80% acetonitrile in 10 mM HCl
gradient:	0-60% buffer B in 5 column volumes

After application of the pH pools, the column is washed with buffer A. During the elution, fractions of 200 ml are collected. Aliquots of the fractions are tested in a bioassay. The fractions are freeze-dried and stored at -20 °C.

Semipreparative reversed-phase C18 chromatography

Fractions 11 and 12 from pH pool V, which had been biologically active in the assay, were separated through a semipreparative reversed-phase column. Fractions 21 to 25 contained the substance according to the invention.

Conditions of chromatography:

column:	4.7 cm x 30 cm steel column
column material:	Vydac RP-C18 15-20 µm, 300 Å
buffer A:	0.1% TFA
buffer B:	0.1% TFA, 80% acetonitrile
gradient:	5-50% B in 45 min, 50-100% B in 10 min
flow rate:	42 ml/min
detection:	214 nm and 280 nm
chromatographic plant:	BioCad
fractions:	every 1.5 min from the start of the gradient

Semipreparative reversed-phase C18 chromatography

The biologically active fractions 21 to 25 from the preceding chromatography were separated through the same semipreparative reversed-phase column. However, methanol was used as the eluent. Fraction 24 contained the substance according to the invention.

Conditions of chromatography:

column:	4.7 cm x 30 cm steel column
column material:	Vydac RP-C18 15-20 µm, 300 Å
buffer A:	0.1% TFA, 20% methanol

buffer B: 0.1% TFA, 100% methanol

gradient: 0-20% B in 6.5 min, 20-80% B in 55 min, 80-100% B in 13 min

flow rate: 30 ml/min

detection: 214 nm and 280 nm

chromatographic plant: BioCad

fractions: every 1.5 min from the start of the gradient

Cation exchange chromatography

The biologically active fractions 19 and 20 from the preceding chromatography were separated through a cation-exchange column. Fractions 45 to 47 contained the substance according to the invention.

Conditions of chromatography:

column: 1 cm x 5 cm steel column

column material: Pepkat, Biotek 5 µm, 300 Å

buffer A: 20 mM sodium phosphate, pH 3.0

buffer B: 20 mM sodium phosphate, pH 3.0, 1.5 M NaCl

gradient: 0-50% B in 50 min, 50-100% B in 10 min

flow rate: 3 ml/min

detection: 280 nm

chromatographic plant: BioCad Sprint

fractions: every 1.5 min from the start of the gradient

Analytical reversed-phase chromatography

The biologically active fractions 45 to 47 from the preceding chromatography were successively separated in several identical runs through a reversed-phase column. Fraction 56 contained the substance according to the invention.

Conditions of chromatography:

column:	1 cm x 25 cm steel column
column material:	Vydac RP-C18 5 µm, 300 Å
buffer A:	0.1% TFA
buffer B:	0.1% TFA, 80% acetonitrile
gradient:	5-50% B in 45 min, 50-100% B in 10 min
flow rate:	2 ml/min
detection:	220 nm
chromatographic plant:	Kontron
fractions:	every 1 min from the start of the gradient

Second analytical reversed-phase C18 chromatography

The biologically active fraction 56 from the preceding separation step was further purified on an analytical reversed-phase column.

Conditions of chromatography:

column:	0.46 cm x 25 cm steel column
column material:	YMC RP-C18, 5 µm, 300 Å
buffer A:	0.1% TFA
buffer B:	0.1% TFA, 80% acetonitrile
gradient:	15-50% B in 75 min, 75-100% B in 10 min
flow rate:	0.7 ml/min
detection:	214 nm
chromatographic plant:	Kontron

Third analytical reversed-phase C3 chromatography

Part of the biologically active fraction 45 from the preceding separation step was directly subjected to mass and sequence analyses. Another part was reduced and alkylated (as described under Example 2) and then further purified on an analytical reversed-phase column.

Conditions of chromatography:

column: 0.1 cm x 15 cm steel column
column material: Zorbax RP-C3, 5 µm, 300 Å

Mass determinations

All mass determinations of the unmodified and modified peptides were performed on a MALDI TOF mass spectrometer. The molecular masses of the peptides were determined as:

IGF-II: 7,471 Da;
IGFBP-2: 12,681 Da;
IGFBP-2: 12,865 Da.

Determination of cysteines/modification of peptides

Cysteines can be detected after previous chemical derivatization, for example, after reduction with β-mercaptoethanol and carboxamidomethylation with iodoacetamide, in the peptide sequencing. Derivatization is followed by desalting, preferably through analytical reversed-phase chromatography on a Vydac RP-C18 column (4.6 mm x 25 cm). Part of the thus modified peptides are subjected to sequence analysis, and mass determinations performed on the rest yield a corresponding molecular mass. From the mass difference to the native peptide, it is concluded that the peptides from hemofiltrate contain six cysteines which are additionally interconnected by three disulfide bridges.

Sequence determination

Both the purified native and the carboxamidomethylated peptides are analyzed by Edman degradation on an ABI 473 A Sequencer using the standard program.

The samples are applied to a Polybrene membrane in amounts of between 100 and 400 pmol. In accordance with the results of mass determinations, the following N-terminal sequences were found:

IGFBP-2-13, MW 12,681
(reduced molecule modified with iodoacetamide, MW 13,045)
Amino acids
GGKHHLGLEEPKKLRPPPARTPCQQELDQV...

IGFBP-2-13, MW 12,865
(reduced molecule modified with iodoacetamide, MW 13,223)
Amino acids
GKGGKHHLGLEEPKKLRPPPARTPCQQELDQV...

IGF-II, MW 7471
Amino acids
AYRPSETLCGGEL....

The C terminus was determined by comparing the measured molecular mass with the mass calculated from the sequence. The identity of these masses is within the measuring accuracy of the MALDI TOF mass spectrometer, i.e., 0.1% of the total mass.

Data base comparison

A data base comparison was performed on the SwissProt and EMBL nucleic acid data bases using the HUSAR program package. The peptide sequences have 100% identity with the amino acids of human IGFBP-2 as derived from the cDNA or with the amino acid sequence of IGF-II.

To date, IGFBP-2 has been described as a 34 kD binding protein the sequence of which was completely analyzed by analyzing the related cDNA (Binkert, C., et al., EMBO Journal Vol. 8 (1989), pages 2497 to 2502). In contrast, IGF-II and also IGF-I, which also binds to IGFBP-2, have already been extensively described in terms of their structures on the protein and DNA sequence levels (as a review: Rechler, M.M., & Nissley, S.P. (1990) "Insulin-like growth factors" in: Peptide growth factors and their receptors (Spori, M.B., Roberts, A.B. eds.), pages 263 to 367, Springer-Verlag, Berlin).

Example 2

Determination of the biological activity of IGF/IGFBP-2-13

The isolation of IGF/IGFBP-2-13 was based on its biological activity in a survival assay of the PC-12 (pheochromocytoma cells) cell line. Thus, aliquots of each of the individual chromatographic stages described under Example 1 were freeze-dried and then subjected to a biological assay. The fractions which gave a positive signal were respectively subjected to further purification.

The assay measures the survival of the cells after having been kept in serum-free medium by determining the activity of mitochondrial enzymes 24 hours after serum starvation. As a positive control, nerve growth factor (NGF) or fetal calf serum (FCS) are employed in this assay.

In 96-well plates, 10,000 PC-12 cells per well were seeded in a serum-free medium, following by the addition of aliquots (about 100 ml equivalent of starting material) to the wells. The survival rate of the cells is measured 20 hours later using a Wst-1 substrate. This substrate is converted by mitochondrial enzymes. The color intensity obtained is measured at 405 nm in an ELISA reader; the reference wavelength was over 600 nm.

The IGF/IGFBP-2-13 complex has a dose-dependent survival-promoting effect on PC-12 cells. These cells correspond to neuronal precursor cells so that it can be considered that IGF/IGFBP-2-13 is a neuroprotective factor.

Example 3

Purification of the peptide IGFBP-4-11 according to the invention

The purification of the peptide IGFBP-4-11 according to the invention was performed by complete analogy with the purification of IGFBP-2-13 described under Example 1 up to the step of the second preparative separation. The further purification was effected by:

Analytical reversed-phase C18 chromatography

Fraction 33 from pH pool IV, which was found biologically active in the assay, was separated through an analytical reversed-phase column. Fraction 34 contained the substance according to the invention.

Conditions of chromatography:

column:	1 cm x 25 cm steel column
column material:	Vydac RP-C4 5 µm, 300 Å
buffer A:	0.1% TFA
buffer B:	0.1% TFA, 80% acetonitrile
gradient:	0-80% B in 80 min, 80-100% B in 10 min
flow rate:	2.5 ml/min
detection:	230 nm
chromatographic plant:	Kontron
fractions:	every 1 min from the start of the gradient

Mass determinations

The mass determinations were performed on an electrospray mass spectrometer. The molecular mass of the peptide was determined as:

IGFBP-4-11, 11,344 Da.

Sequence determination

The amino acid sequence of the purified native biologically active peptide IGFBP-4-11 was determined as described under Example 1.

The following N-terminal sequence was found:

IGFBP-4-11, MW 11,344 Da

KVNGAPREDARPVPQGSXQSELIIRALERL...

The C terminus was determined by comparing the measured molecular mass with the mass calculated from the sequence. The identity of these masses is within the measuring accuracy of the electrospray mass spectrometer, i.e., 0.1% of the total mass.

Data base comparison

A data base comparison was performed on the SwissProt and EMBL nucleic acid data bases using the HUSAR program package. The peptide sequence has 100% identity with the amino acids of human IGFBP-4 as derived from the cDNA.

Determination of the sulfur bridge cross-links in IGFBP-4-11

The analysis of the sulfur-bridge cross-linking was performed by cleaving the native peptide IGFBP-4-11 in two different parallel reactions with the endoproteases chymotrypsin and Arg-C. The cleaving fragments obtained were then separated by analytical reversed-phase chromatography and subjected to molecular mass and sequence analyses. The following fragments containing two cysteines and one sulfur bridge each were obtained:

HPKQCHPALDGQRGKCW, MW 1960

CVDRKTGVKLPGGLEPKGELDCHQLADSF, MW 3112

PVPQGSCQSELHR

MW 3236

THEDLYIIPIPNCDR

It can be seen therefrom that the sulfur bridges between cysteines 1 and 2, between cysteines 3 and 4 and between cysteines 5 and 6 are realized in the native IGFBP-4-11.

Example 4

Determination of the biological activity of IGFBP-4-11

The isolation of IGFBP-4-11 was based on its biological activity in a proliferation assay with primary bone cells (osteoblasts), which were first isolated from rat embryonal calvarias.

Thus, aliquots of each of the individual chromatographic stages described under Example 3 were freeze-dried and then subjected to a biological assay. The fractions which gave a positive signal were respectively subjected to further purification. The assay measures the proliferation of the cells by determining the incorporation of radioactive thymidine, i.e., the DNA synthesis rate, 48 or 72 hours after the addition of the fractions. As a positive control, transforming growth factor-beta (TGF-β) or fetal calf serum (FCS) are employed in this assay. In 96-well plates, 5000 osteoblasts per well were seeded in a serum-containing medium, following by the addition of aliquots (about 100 ml equivalent of starting material) to the wells. The proliferation rate (DNA synthesis rate) of the cells is measured 48 or 72 hours later using the addition and incorporation of radioactive thymidine. The peptide IGFBP-4-11 has a dose-dependent proliferation-promoting effect on these primary osteoblasts. These cells correspond to typical bone cells so that it can be considered that IGFBP-4-11 is an osteoanabolic factor.

Example 5

Isolation of the C-terminal domain of IGFBP-3

By a similar method to that used in Examples 1 and 3, a peptide could be isolated from hemofiltrate, having a mass of 2,470 Dalton (MALDI: 2481 Dalton) and the following sequence:

HTRISELKAEAVKKDRRKKLTQS (?)

from which the following sequence results as the C-terminal sequence of IGFBP-3:

KVDYESQSTDTQNFSSESKRETEYGPCRREMEDTLNHLKFLNVLSPRGVHIPNCDKK
GFYKKKQCRPSKGRKRGFCWCVDKYGQPLPGYTTKGKEDVHCYSMQSK

Example 6

By a similar method to that used in Examples 1 and 3, the N-terminal domain of IGFBP-4 could be isolated, having the following sequence:

DEAIHCPPCSEEKLARCRPPVGCEELVREPGCGCCATCALGLGMPCGVYTPRCGSGLRCYP
PRGVEKPLHTLMHGQGVCMELAEIEAIQESLQPSDKDEGDHPNNSFSPCSAHDRRCLQKH
FAKIRDRSTSGGKM

Example 7

Determination of the C-terminal sequence of IPB-5

By a method as in Examples 1 and 3, a peptide with a mass of 13.5 kD could be determined. The sequence determination gave the following sequence:

KFVGGAENTAHPRIISAPEMRQESEQGPCRRHMEASLQELKASPRMVPRAVYLPNCDRKG
FYKRKQCKPSRGRKRGICWCVDKYGMKLPGMEYVDGDFQCHTFDSSNVE

The theoretical mass is 12.5 kD, and therefore, it has to be considered that the peptide is glycosylated at serine or threonine.